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Study of LDL and acetylated LDL endocytosis by mononuclear cells in HIV infection

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Abstract

Activated lymphocytes have a high level of low density lipoprotein (LDL) uptake as compared to resting lymphocytes, whereas scavenger receptors for acetylated LDL (Ac-LDL) are expressed on limited number of immune cells, i.e., monocytes/macrophages. The endocytosis of LDL and Ac-LDL by mononuclear cells was studied during in vitro and in vivo HIV infection, in order to use LDL and Ac-LDL as carriers of antiviral and/or immunomodulatory drugs towards lymphocytes and monocytes. The uptake of LDL and Ac-LDL was analyzed by cytofluorimetry. LDL endocytosis in PHA/IL2-activated lymphocytes was higher than in resting lymphocytes. In vitro HIV infection of PHA/IL2-activated lymphocytes did not alter the high LDL endocytosis in lymphocytes, $CD4^+$ and $CD8^+$ cells. In a group of 12 symptomatic patients there was no alteration of LDL endocytosis in lymphocytes, CD4 and CD8 lymphocytes. In another group of 23 individuals, the Ac-LDL endocytosis mediated by $CD14^+$ monocytes was unaltered in asymptomatic patients ($n = 6$) and in some symptomatic patients ($n = 6$, $CD14^+$ cells $> 100/mm^3$). On the contrary, in other symptomatic patients ($n = 11$, $CD14^+$ cells $< 100/mm^3$), the number of Ac-LDL^+ $CD14^+$ cells decreased, whereas their efficiency of Ac-LDL endocytosis increased as compared to those of other HIV^+ patients. In conclusion, the use of lipoproteins as carriers to increase the drug delivery to $CD4^+$ lymphocytes and to $CD14^+$ monocytes can be envisaged, since: (i) the LDL endocytosis was not impaired in CD4 lymphocytes of HIV^+ patients, and (ii) the Ac-LDL uptake by monocytes was altered only in some patients of stage IV.

Keywords: LDL; CD4 Lymphocyte; Monocyte; HIV

1. Introduction

The low density lipoproteins (LDL) are small natural vesicles surrounded by a monolayer of phospholipid in which a specific protein, apolipoprotein B (apo B), is embedded. The LDL deliver cholesterol to the cell through endocytosis mediated by the high affinity receptors which react with the apo B. Although LDL receptors are found on the surface of every cell, their expression differs widely and this differential expression can be used as a basis for cell targeting. Thus, previous data have shown that cytotoxic compounds incorporated into LDL can be favourably delivered to tumour cells by the LDL pathway, since the specific endocytosis of LDL increases in numerous tumours in vitro and in vivo [1]. On the other hand, high affinity LDL receptors are present on lymphocytes [2]. Consequently, the LDL pathway might also be used for carrying antiviral and/or immunomodulatory compounds to lymphocytes and especially to activated lymphocytes that can benefit by the high level of LDL receptor expression induced by cell activation. Indeed, it has been demonstrated that T lymphocytes activated by Mab CD3 + IL2 have high LDL receptor expression as compared to resting T cells [3]. Such a therapeutic approach could be efficient during HIV infection. Indeed, HIV can enter in resting T lymphocytes and initiate viral DNA synthesis with the same efficiency as in stimulated cells; however, only activated lymphocytes can produce virus [4]. Thus, $CD4^+$ lymphocytes must be activated to spread viral infection.

In addition, scavenger receptors for modified LDL have been described at the surface of human macrophages but not on other immune cells [5]. The scavenger receptors

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bind negatively charged lipoproteins and participate in the removal of altered lipoproteins from the circulation. Since LDL can be easily altered at the apolipoprotein B (apo B) site by chemical agents such as acetic acid [6], acetylated LDL can be used to carry drugs specifically towards monocyte-derived cells. In HIV infection monocytes/macrophages are a main reservoir of the virus [7,8]. Recently, covalent coupling of nucleosides to apo B have been performed, and, by altering the apo B, have allowed their targeting to mice macrophages in vitro through the scavenger receptor [9]. However, a prerequisite for the use of LDL and acetylated LDL (Ac-LDL) to increase the delivery efficiency of anti-HIV and/or immunomodulatory drugs to immune cells is the knowledge of specific receptor activities in HIV-infection conditions. The purpose of this work was to investigate the activity of LDL receptors in lymphocytes and of Ac-LDL receptors in monocytes during in vitro and in vivo infection by HIV-1.

2. Materials and methods

2.1. Patients

Total blood was obtained from both HIV-seronegative healthy donors ($n = 15$) and HIV-infected donors ($n = 35$) from the outpatients' department of Purpan hospital (Toulouse, France). HIV-infected patients were classified according to the recommendations of Centers for Disease Control (CDC): 6 asymptomatic patients (CDC stages II and III), 29 symptomatic patients (CDC stage IV).

2.2. Isolation, activation and infection of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy HIV seronegative donors by centrifugation through Ficoll Ipaque (Pharmacia, Guyancourt, France). PBMC ($2 \cdot 10^6$ cells/ml) were cultured for 48 h with phytohemaglutinin (PHA, 3 μg/ml; Sigma, La Verpillière, France) in RPMI 1640 medium supplemented with Glutamine 2 mM, Fetal Calf Serum 20% (Gibco-BRL, Cergy Pontoise, France), Penicillin, Neomycin, Streptomycin and Interleukin 2 (IL2, 10 I.U./ml). The day of infection was defined as Day 0 (D0). After washing, PHA-activated PBMC ($60 \cdot 10^6$) were infected 2 h at 37° C with 3 ml of supernatant containing HIV-1 BRU (Pasteur, Paris, France, TCID 50:1000/ml). The cells were washed and cultured in a fresh medium + IL2 ($2 \cdot 10^6$ PBMC/ml) for several days. No fresh lymphocytes were added to the culture, but half the culture medium was changed every four days. The control culture consisted of non-infected cells of the same donor. The infection progression was assessed by evaluating the p24 antigen level by ELISA (Abbott, Rungis, France) in the cell-free supernatant and in the cell pellet. In long term culture, before each experiment the cell viability was around 80% as determined by the Trypan blue exclusion test.

2.3. Lipoprotein isolation and labelling with the fluorescent probe DiI

Low-density lipoproteins (LDL, $d = 1.019 - - 1.063$ g/ml) were isolated from the plasma of healthy volunteers by sequential flotation in KBr (potassium bromide) as previously described [10]. The concentration of protein in LDL was determined by the method of Lowry. LDL was labelled with 1,1'-dioctadecyl-3,3'tetramethylindocarbocyanine perchlorate (DiI, Interchim, Montluçon, France) as described [11]. In some experiments, labelled and unlabelled lipoproteins were acetylated with repeated addition of acetic anhydride [6]. Acetylated LDL were characterized by electrophoresis (Tris-Veronal buffer, pH 8.6) in 1% gel agarose to evaluate increased migration due to the modification of the whole lipoprotein particle charge [6]. DiI-labelled LDL were previously used to study LDL receptor-mediated endocytosis of lymphocytes [3,12], whereas DiI-AcLDL were used for studying the Ac-LDL uptake by monocytes [12–14]. Fluorescent labelled cells can be analyzed by a fluorescence-activated cell sorter (FACS). In addition, FACS allows an easy study of specific cell subpopulations after staining by fluorescent monoclonal antibodies.

2.4. Uptake of DiI-labelled LDL (DiI-LDL) and of acetylated DiI-labelled LDL (DiI-AcLDL) by infected and uninfected cells

Routinely, $1 \cdot 10^6$ PBMC from infected or non-infected cultures taken at different days after infection were incubated with different concentrations (3 to 50 μg/ml) of DiI-LDL for 5 h at 37° C. For experiments done with cells of the HIV^+ patients, total blood was washed twice with RPMI – 1% BSA; 200 μl of blood was then incubated for 5 h at 37° C with 50 μg of DiI-LDL or for 3 h with 10 mg of DiI-AcLDL in a final volume of 1 ml of RPMI-1% BSA. High-affinity LDL or Ac-LDL uptakes were determined with low concentrations of labelled lipoproteins in the absence (total uptake) or presence (non-specific uptake) of a 20-fold excess of unlabelled LDL or of a 60-fold excess of unlabelled Ac-LDL as we have previously described [12]. Cells were then washed at 4° C with RPMI-1% BSA. In some experiments (50 μg/ml of DiI-LDL or DiI-AcLDL, 5 h, 37° C, $n = 3$), the total cellular content of DiI in freshly $CD3^+$ lymphocytes and in freshly $CD14^+$ monocytes of healthy donors was measured using a fluorimetric method [15]. The total cellular content of DiI was 12 ng/mg of proteins in lymphocytes and 160 ng/mg of proteins in monocytes, i.e., in term of apo B: respectively, 240 ng/mg of proteins and 3.2 μg/mg of proteins.

2.5. Immunologic staining of cell suspension

Infected and uninfected cells that internalized labelled lipoproteins were stained with anti-CD3, anti-CD4, anti-CD8 and anti-CD14 monoclonal antibodies (MAbs) (2 $\mu g/10^6$ cells, Immunotech, France) coupled with fluorescein isothiocyanate. In experiments conducted with total peripheral blood, the red blood cells were lysed for 5 min at room temperature with lysing reagent (Immunotech, Marseille, France). Cells were fixed overnight with PBS-2% paraformaldehyde, then analysed by a two-colour analytical procedure in a cytofluorimeter.

2.6. Flow cytometry analysis

Analysis was performed either with the Fluorescence-activated cell sorter (FACS, Becton Dickinson) or with an Epic Elite (Coulter), both equipped with an argon ion laser. The laser was operated in the 488 emission wavelength. Forward angle and 90° light scatter gates were established to exclude dead cells and cell debris from analysis. Routinely, the lymphocyte and the monocyte populations were selected on the basis of cell size, cell structure and positivity either to MAb anti-CD3 or to MAb anti-CD14. Fluorescence (> 570 nm on the FACS or > 575 nm band pass on the Epic Elite) signals from the accumulated DiI in the cells were collected by the red photomultiplier, converted to digital format and processed for storage and display with 256 or 1024 channel resolution (FACS and Epic Elite, respectively) in one-parameter logarithmic scale frequency histograms. For experiments done with cultured cells, five thousand cells were analysed for each sample. In experiments conducted with the cells of HIV-infected patients, because of the low number of monocytes and lymphocytes in some patients, not less than 20 000 cells were analysed. Autofluorescence of cells incubated with unlabelled lipoproteins was used as the negative control in each experiment. The percentage of cells that take up lipoproteins was determined by counting the labelled cells with DiI which exceeded the upper limit of autofluorescence of the control cells. The percentage of fluorescence positive cells that specifically internalized the DiI-lipoproteins was calculated by subtracting the non-specific positive cells that take up DiI-lipoproteins in presence of an excess of unlabelled lipoproteins (see above). Data were also expressed as mean red fluorescence intensity (MFI) in arbitrary units (AU). In some experiments, the specific LDL uptake in the lymphocyte population was expressed as specific total internalization (TI) calculated as follows: percentage of specific DiI-positive cells × MFI.

2.7. Statistics

Statistical analysis of data was carried out using Student's *t*-test and non-parametric Mann–Whitney *U*-test. *P*-values were calculated for assessment of significance.

3. Results

3.1. Effect of in vitro HIV infection on LDL uptake by lymphocytes

In a first set of experiments the effect of PHA and IL2 on the LDL uptake by lymphocytes was studied. As shown in Fig. 1, IL2 alone cannot efficiently stimulate the LDL uptake by lymphocytes, but PHA-activation strongly increased the LDL endocytosis as compared to PHA-un-

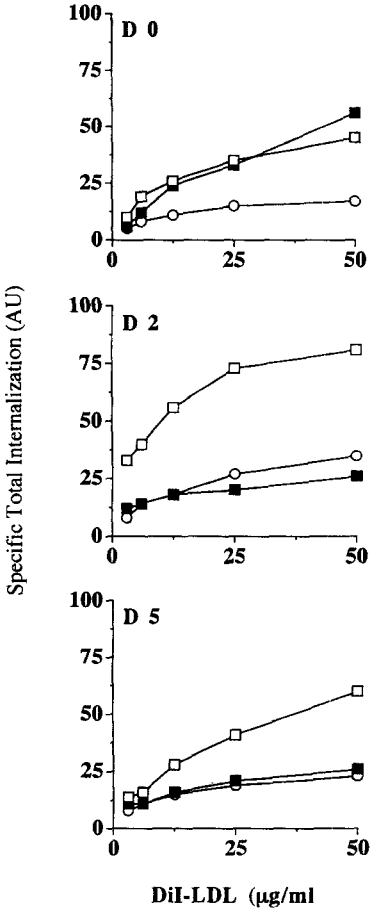

Fig. 1. Effect of cellular activation on LDL endocytosis by lymphocytes. PBMC from healthy donors were cultured for 48 h with IL2 (10 I.U./ml) + PHA (3 μg/ml) (□), with IL2 (■). After washing (day 0), cells were cultured for an additional 5 days in medium with IL2. Aliquots of cells were cultured (48 h + washing + 5 days) in medium alone (○). At days 0, 2 and 5, 10^6 PBMC were harvested, washed and incubated with different concentrations of DiI-LDL for 5 h at 37° C. Non-specific endocytosis was performed in the presence of 20-fold excess of unlabelled LDL. For each sample, $5 \cdot 10^3$ cells were analysed by flow cytometry in lymphocyte area selected as described in Section 2. The percentage of LDL positive cells obtained after substraction of non-specific LDL positive cells, and their mean fluorescence intensity (MFI) in arbitrary units (A.U.) were determined. The total internalisation (TI) which represents LDL specific endocytosis in the lymphocyte population was calculated as follows: TI = % of DiI-LDL$^+$ lymphocytes × MFI. The results are the arithmetic mean of the duplicates and are representative of two identical experiments.

treated cells at days 2 and 5. It appears that PHA/IL2-treated cells took up LDL about 2- to 3-times more than unactivated cells and IL2-treated cells.

In subsequent experiments, PHA/IL2-activated PBMC were infected by HIV-1 BRU, harvested at different days and incubated with DiI-LDL. When observed at day 2 after infection, lymphocytes took up LDL as efficiently as uninfected cells either in LDL dose–effect assays or in time-effect assays. For example, in one experiment after a 5 h incubation with 3 and 50 μg/ml of DiI-LDL, 18% and 70% of lymphocytes of infected culture took up LDL (MFI = 0.5 and 0.6 A.U.) versus 20% and 70% of DiI-LDL$^+$ lymphocytes (MFI = 0.5 and 0.7 A.U.) in control culture. Similarly, after 0.5, 1, 2, 4 and 5 hours of incubation with 50 μg/ml DiI-LDL, 12, 26, 33, 55 and 64% of lymphocytes of infected culture took up LDL (MFI = 0.5 to 0.6 A.U.) versus 13, 25, 35, 57 and 60% of DiI-LDL$^+$ lymphocytes (MFI = 0.5 to 0.6 A.U.) in control culture. According to the day of culture, the LDL total internalization (TI) in lymphocytes of infected cultures was similar to that of non-infected cultures (Fig. 2A). Thus, HIV infection does not disturb the DiI-LDL total endocytosis mediated by the PHA/IL2-activated lymphocyte population. Results of a dual immunofluorescence analysis showed that the same percentage of CD4$^+$ and CD8$^+$ cells were able to take up DiI-LDL in each T-lymphocyte population (Fig. 2B). Identical results were observed in long-time cultures (18 days). Viral infection was controlled by determining the level of p24 antigen concentration in the supernatant of culture: p24 antigen increased steadily from day 0 to day 18 (Fig. 3A). As expected, since CD4$^+$ cells represent the main target cell of HIV infection [16], CD4$^+$ lymphocyte percentage decreased as viral antigen concentration increased (Fig. 3B). The specific LDL uptake by lymphocytes, expressed as TI, was determined at different days after infection. The ratio of TI in lymphocytes of infected culture/TI in lymphocytes of non-infected culture was then calculated. As shown in Fig. 3 C, this ratio is around 1. A dual immunofluorescence analysis showed that CD4$^+$ and CD8$^+$ cells from infected culture take up LDL as efficiently as cells from control culture since the MFI of DiI-positive cells is identical in infected and uninfected culture (Fig. 3D and E). The same results were observed when the results were expressed as percent of DiI-LDL$^+$ cells in each T-cell populations (not shown). These results indicate that whatever the progression of in vitro HIV infection and the CD4 cell number, the ability of PHA/IL2 activated lymphocytes to endocytose LDL is unaltered by HIV.

3.2. LDL uptake by lymphocytes of HIV-1 infected patients

LDL endocytosis by lymphocytes was studied in infected patients of stage IV ($n = 12$). The high specific DiI-LDL uptake was assessed in the total lymphocyte population and in CD4$^+$ or CD8$^+$ lymphocyte subpopulations. Fig. 4 shows that total lymphocytes, CD4$^+$ and CD8$^+$ lymphocytes of HIV-1 infected patients endocytosed LDL as efficiently as lymphocytes of non-infected subjects. Then, the previous HIV$^+$ patients were separated into two groups depending on the usual limit of 200 CD4$^+$ cells/mm^3 (< 200 CD4$^+$ cells/mm^3, $n = 8$; > 200 CD4$^+$ cells/mm^3, $n = 4$). When results were expressed as percent of DiI-LDL$^+$ cells and as MFI in each T-cell subpop-

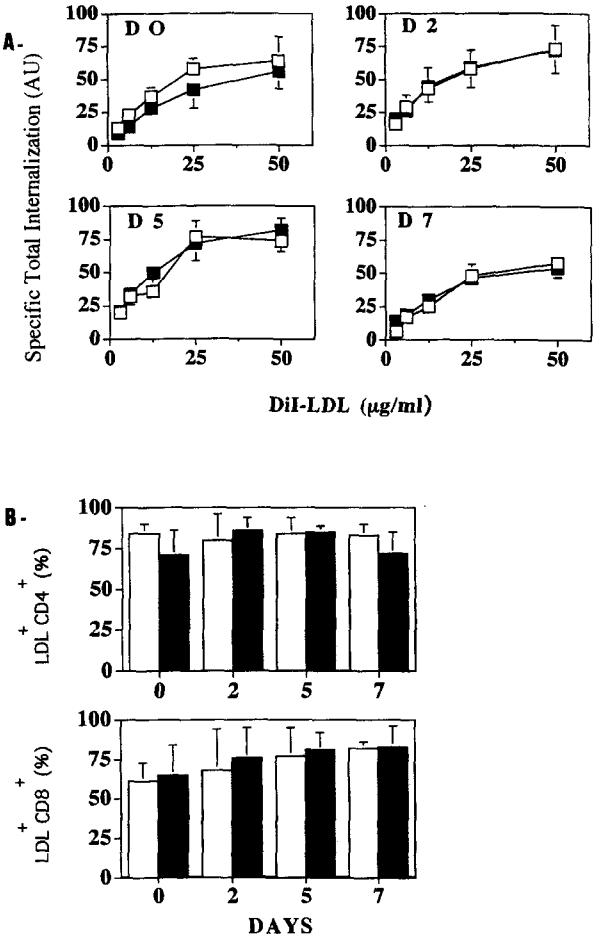

Fig. 2. Effects of in vitro HIV infection on LDL endocytosis by lymphocytes in short-term culture. PMBC were activated with PHA + IL2 for 48 h. Cells were washed and infected (■) or not (□) with HIV-1 at day 0, then cultured for 7 days in a medium containing IL2. (A) At different days after infection, 10^6 cells were collected, washed and incubated with different concentrations of DiI-LDL for 5 h at 37° C. Non-specific endocytosis was performed in the presence of 20-fold excess of unlabelled LDL. Simple histogram analysis was performed in lymphocyte area to determine the percentage of DiI-LDL$^+$ cells. Results are expressed as total specific internalization of DiI-LDL in lymphocyte population from day 0 (D0) to day 7 (D7). (B) At different days after infection, cells were incubated for 5 h with 50 μg of DiI-LDL, then stained with anti-CD4 and anti-CD8 MAbs. A dual fluorescence analysis was assessed to determine the percentage of LDL$^+$ CD4$^+$ or LDL$^+$ CD8$^+$ cells. The figures represent the % of LDL$^+$ CD4$^+$ in CD4$^+$ lymphocyte population (B Top) and the % of LDL$^+$ CD8$^+$ in CD8$^+$ lymphocyte population (B Bottom) after substraction of non-specific DiI-LDL endocytosis. The results shown in A and B are mean ± S.D. of 3 independent experiments.

ulation, LDL endocytosis was similar in the both groups of HIV$^+$ donors (not shown).

3.3. Modified LDL uptake by monocytes of HIV-1 infected patients

The activity of Ac-LDL receptors on the monocyte surface of HIV-1 infected patients was studied through

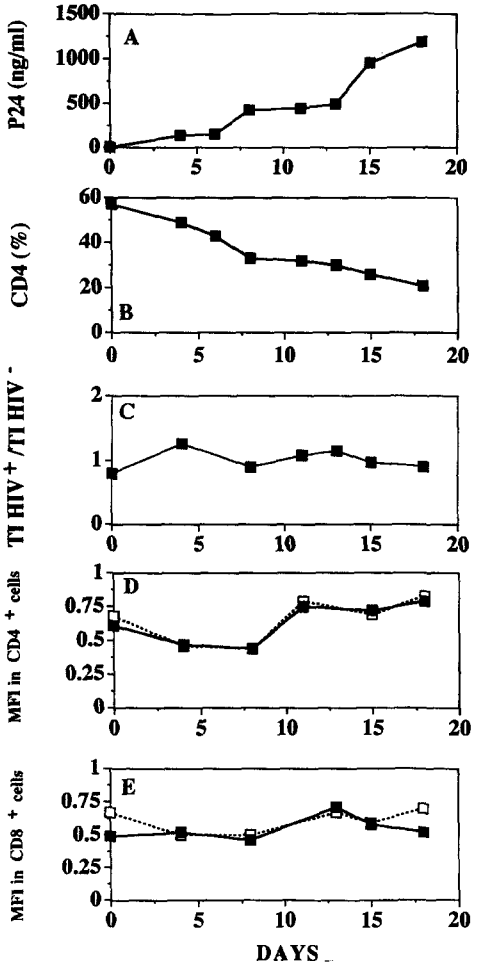

Fig. 3. Effects of in vitro HIV infection on LDL endocytosis by lymphocytes in long-term culture. Activated PBMC were infected (day 0) with HIV-1, washed and cultured for 18 days in a medium containing IL2. At different days after infection, aliquots of cells were harvested and stained with MAb anti-CD4. Other aliquots were incubated with 50 μg of DiI-LDL for 5 h at 37° C. Non-specific endocytosis was performed in the presence of 20-fold excess of unlabelled LDL. Then for each sample incubated with lipoproteins, cells were stained with anti-CD4 and anti-CD8 MAbs. A simple histogram and a dual fluorescence analysis were performed. Viral infection was assessed by ELISA for determination of p24 viral antigen concentration in the supernatant of culture. Percentage of specific LDL$^+$ lymphocytes of infected and non-infected cultures and their MFI was determined. From the top to the bottom: (A) p24 antigen concentration (ng/ml) assessed by ELISA test at different days after infection, (B) percent of CD4$^+$ cells in infected culture, (C) LDL-specific total internalization (TI) in lymphocytes of infected culture/LDL TI in lymphocytes of non-infected culture, (D) and (E) MFI in arbitrary units of LDL$^+$ cells in CD4$^+$ and CD8$^+$ lymphocyte populations of infected (■) or non-infected culture (□). Results are representative of two identical experiments.

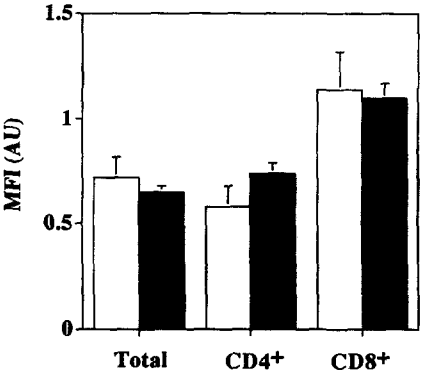

Fig. 4. LDL endocytosis by lymphocytes of HIV infected patients. Washed samples of 200 μl of blood from 12 HIV$^+$ patients, classified in stage IV according to the CDC classification, were incubated with 50 μg of DiI-LDL for 5 h at 37° C. Non-specific endocytosis was performed in the presence of a 20-fold excess of unlabelled LDL. Aliquots of each sample incubated with lipoprotein were stained with anti-CD4 MAb and anti-CD8 MAb conjugated with FITC. Red cells were lysed, then a simple histogram analysis and a dual fluorescence analysis were performed. Results represent LDL endocytosis expressed as mean fluorescence intensity (MFI) ± S.E.M., in total lymphocytes (Total), CD4$^+$ and CD8$^+$ lymphocytes of HIV-1 infected patients, $n = 12$ (■) or non-infected controls, $n = 5$ (□).

experiments of AcLDL endocytosis. As for LDL uptake, competitive assays with unlabelled Ac-LDL were done to determine high specific Ac-LDL uptake. Firstly, Ac-LDL endocytosis by monocytes from blood of healthy donors was compared to that of lymphocytes from the same donors. After a 5 h incubation of cells with 50 μg/ml of DiI-AcLDL, a dual fluorescence analysis of DiI-AcLDL endocytosis by freshly isolated CD14$^+$ monocytes or freshly isolated CD3$^+$ lymphocytes was performed. As expected, CD14$^+$ monocytes have a high AcLDL endocy-

Table 1
Ac-LDL uptake by monocytes from HIV-infected patients

Patients	Percentage	MFI
Controls ($n = 10$)	85 ± 12	6.5 ± 2.3
Asymptomatics ($n = 6$)	83 ± 10	5.2 ± 3.3
Symptomatics [a] ($n = 6$)	81 ± 7	4.6 ± 2.0
Symptomatics [b] ($n = 11$)	66 ± 21 *	8.9 ± 2.1 *,○,●

Washed blood samples were incubated with 10 μg of Ac-LDL for 3 h at 37° C. Non-specific endocytosis was performed in the presence of a 60-fold excess of unlabelled Ac-LDL. After staining by anti-CD14 MAb, red cells were lysed and Ac-LDL uptake by CD14$^+$ monocytes was assessed by flow cytometry. The results (mean ± S.D.) are expressed as percentage of DiI-AcLDL positive cells after subtraction of non-specific endocytosis and as MFI in A.U. Controls were HIV seronegative healthy donors ($n = 10$). HIV$^+$ infected patients ($n = 23$) were classified according to their clinical stage as defined by the CDC: asymptomatic HIV$^+$ patients (stage II and III) and symptomatic HIV$^+$ patients (stage IV). In symptomatic patients, two groups were formed according to their CD14$^+$ count/mm^3: [a] CD14 > 100, $n = 6$; [b] CD14 < 100, $n = 11$. * $P < 0.05$ as compared to the controls, ○ $P < 0.05$ as compared to asymptomatic HIV$^+$ patients, ● $P < 0.01$ as compared to symptomatic patients of group a (Student's t-test; in each case, the significance of the differences was confirmed by Mann–Whitney U-test).

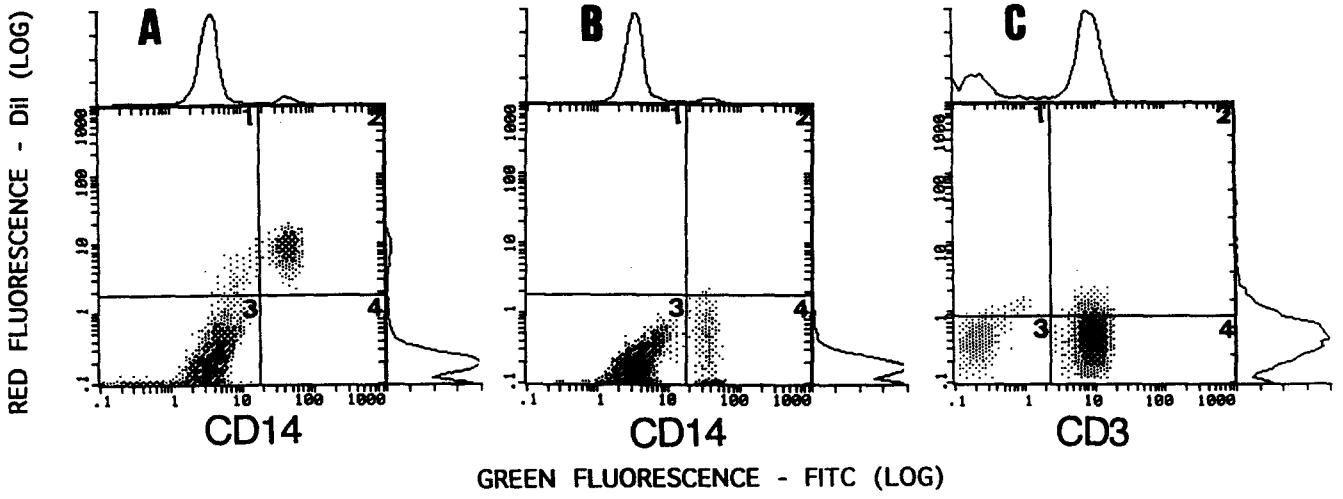

Fig. 5. Dot plots of dual-colour immunofluorescence of DiI-AcLDL endocytosis by $CD14^+$ monocytes or $CD3^+$ lymphocytes. Total blood from healthy donors was washed and 200 μl of blood were incubated with 50 μg/ml DiI-AcLDL for 5 h at 37° C. Cells were then washed and stained by MAb anti-CD14 or MAb anti-CD3 conjugated with fluorescein (FITC). Red cells were lysed and the remaining cells were washed twice and fixed. The figure shows the results obtained from one donor. $CD14^+$ monocytes analysis: cells were gated in an area containing monocytes and lymphocytes. However, the same results were obtained when cells gated only in the monocyte area were analysed. DiI-AcLDL endocytosis by freshly isolated $CD14^+$ monocytes in the absence (A) or in the presence of 600 μg/ml unlabelled Ac-LDL (B). (C) DiI-AcLDL endocytosis in the absence of unlabelled Ac-LDL by $CD3^+$ lymphocytes gated in lymphocyte area. For each dot plot, quadrant 1 represents DiI staining only, quadrant 2 is dual staining, quadrant 4 is fluorescein staining. The unstained cells are in quadrant 3. Same dot-plots were obtained for DiI-AcLDL by $CD14^+$ monocytes from HIV-infected patients.

tosis (Fig. 5A) as compared to $CD3^+$ lymphocytes (Fig. 5C). Competition assays showed that DiI-AcLDL endocytosis by $CD14^+$ monocytes, mediated by scavenger receptor, is strongly inhibited by an excess of unlabelled AcLDL (Fig. 5B). Experiments of dose and time effects with blood of healthy subjects were then performed and showed saturated AcLDL endocytosis at concentrations as low as 10 μg/ml and for 3 h incubation time (results not shown). Finally, these later conditions were used to study AcLDL endocytosis by monocytes of infected patients. The study of the DiI-AcLDL endocytose, expressed as MFI and % of fluorescence positive cells, in $CD14^+$ monocytes of HIV-1 infected patients ($n = 23$) and non-infected controls ($n = 10$) showed no difference (results not shown). In the same way, when infected patients were divided into two groups according to the limit number of 200 $CD4^+$ lymphocytes/mm^3, there was no difference as compared to healthy donors or between the infected patients themselves. However, 10/14 patients with less than 200 CD4/mm^3 had a number of $CD14^+$ monocytes < 100/mm^3 versus only 2/9 in patients with more than 200 $CD4^+$ cells/mm^3. Consequently, HIV^+ patient dispatching was done depending on this arbitrary low limit of $CD14^+$ monocyte number. Under these circumstances, in patients with less than 100 $CD14^+$ cells/mm^3 ($n = 12$) the $AcLDL^+$ $CD14^+$ cell percentage (mean $\pm$ S.D.) was decreased (66 $\pm$ 19%), whereas their MFI was increased (8.6 $\pm$ 2.2%) as compared to controls (respectively 85 $\pm$ 12%, $P < 0.02$ and 6.5 $\pm$ 2.3%, $P < 0.05$) and to the other group of HIV^+ patients (83 $\pm$ 8%, $P < 0.02$ and 4.9 $\pm$ 2.7%, $P < 0.005$). Infected patients were also divided into two groups according to disease expression (Table 1): asymptomatic patients and symptomatic patients. Asymptomatic patients ($n = 6$) had the same Ac-LDL endocytosis as controls, whereas symptomatic patients showed moderate decrease of the $Ac\text{-}LDL^+$ cell percentage ($P < 0.02$ in patients with $CD14^+$ number < 100/mm^3, as shown in Table 1). Finally, the association of clinical and immunological characteristics confirmed that, in symptomatic patients, a low number of CD14 monocytes/mm^3 (< 100) is associated with an increase of the AcLDL uptake efficiency (in MFI) by some of these monocytes (Table 1).

4. Discussion

In this work the effects of HIV-1 infection on the LDL and Ac-LDL endocytosis by lymphocytes and monocytes were studied. Once a DiI-labelled lipoprotein has been internalized by cells, lysosomal enzymes degrade the lipoprotein molecules and DiI accumulates in the membranes. Cells that metabolize lipoproteins at different rates will accumulate varying amounts of DiI and will display different fluorescences. Thus, the increase of fluorescence in PHA/IL2-activated cells as compared to inactivated cells confirms that LDL receptor activity is enhanced by lymphocyte activation and proliferation as previously shown by the use of anti-CD3 MAb + IL2 [3].

Impairment of lymphocyte functions is one of the main characteristics of HIV-1 infection [17]. In in vitro experiments of short-term and long-term culture, we have found that total lymphocytes, $CD4^+$ and $CD8^+$ lymphocytes, activated by PHA/IL2 and harvested from HIV-infected cultures, endocytosed LDL as efficiently as lymphocytes

from uninfected cultures. Although the level of LDL endocytosis directly performed by the HIV-infected $CD4^+$ lymphocytes was not demonstrated, results show that, in infected cultures, the LDL endocytosis by the $CD4^+$ lymphocyte subpopulation, containing the main HIV-target cells and in activation conditions, is not impaired. In experiments with HIV-1 infected patients, a rapid technique was developed to study LDL or Ac-LDL endocytose by either lymphocytes or monocytes using total peripheral blood. As observed in in vitro infection experiments, in vivo infection does not modify the effectiveness of LDL endocytosis by either total peripheral lymphocytes or $CD4^+$ and $CD8^+$ lymphocytes. Although we have not directly studied the LDL receptor activity in lymphocytes which were both HIV-infected and activated in vivo, taken together our in vivo and in vitro results suggest that a high LDL endocytosis can exist in activated CD4 lymphocytes during HIV infection.

Monocytes have important immunoregulatory functions that can be compromised by HIV infection and pathological changes in monocytes can contribute to the impairment of the immune system [18]. However, a lot of clinical studies show little or no change in blood monocyte number, phenotype or functions throughout HIV infection [19]. In the same way, our results indicate that Ac-LDL endocytosis is unaltered in asymptomatic HIV-infected patients. However, the efficiency of Ac-LDL endocytosis clearly increases in some monocytes, from the asymptomatic patients to the symptomatic patients characterized by a high immunological perturbation, suggesting that a progressive change in cell metabolism appears during the infection (Table 1). It is unlikely that this increase of AcLDL endocytosis can be due only to the change of HIV-infected monocyte number. Indeed, there is an exceedingly low frequency of infected monocytes in circulation ($< 0.01\%$ of total monocytes [19]). However, the development of HIV infection can alter some functions of uninfected monocytes. Thus, clinical studies have shown a correlation between the disease stage and the capacity of blood monocytes to produce IFN [19], and recent reports have indicated that HIV glycoproteins, e.g. gp120, can modulate the monocyte secretory function [20]. In the same way, there might be a correlation between the alteration of Ac-LDL endocytosis and the stage of disease.

Over the last 10 years, LDL have been examined experimentally as drug-delivery vehicle [21,22]. Thus, the use of LDL as a carrier of drugs in cancer therapy is well documented [1,23]. Some advantages of the delivery by using LDL and modified LDL could be: (i) an increase in the half-life (2 to 3 days for LDL) of the drug and the targeting towards the cells characterized by high LDL requirements, (ii) a decrease in the risk of inducing immune response to the drug and of its damage by extracellular enzymes, (iii) an easier diffusion from the vascular to the extravascular compartment, (iv) an intracellular uptake of drug via a receptor-mediated endocytosis allowing the bypass of drug resistance mechanisms at the cell level. The main disadvantage of LDL is a lack of targeting, since receptors are expressed on numerous tissues. Thus, the liver takes up about 2/3 of blood LDL. However, as shown in vivo in human cancer, administration of bile acids down-regulates LDL-receptor activity in the liver and increases the targeting towards the tumour [23]. A second disadvantage of LDL as drug delivery system, is their ability to entrap only lipophilic drugs, most drugs used in therapeutics being hydrophilic. However, new drug delivery systems were recently built to mimic LDL and to allow entrapment of hydrophilic drugs [24]. Finally, numerous works have shown the feasibility of preparation of antitumour drug-LDL complexes and the efficiency of their delivery to the tumour with high uptake of LDL [1,23]. Similarly, since lymphocyte proliferation and activation strongly increase LDL uptake in spite of HIV infection, and since HIV replication is strictly dependent on cell activation [25,26], it is reasonable to suggest that a significant targeting of anti-HIV drugs by means of LDL receptor to activated lymphocytes can be obtained.

The apoprotein of LDL can be modified to allow recognition by receptors other than the physiological LDL receptors: e.g., lactosylated LDL allow drug-targeting to Kuppfer cells by galactose-specific receptors [27]. Unlike LDL, the acetylated LDL allows specific targeting to monocytes/macrophages, since they are the only immune cells endocyting acetylated LDL via the scavenger receptors with a very high efficiency [28]. However, the acetylated LDL is removed from the circulation by the sinusoidal endothelial cells of the liver, spleen, bone marrow, adrenal and ovary glands. The other endothelial cells (of arteries, veins, or capillaries of the heart, testes, kidney, brain, adipose tissue, and duodenum) did not endocytose Ac-LDL [29]. The specific uptake of AcLDL by sinusoidal endothelia could reflect the requirement for cholesterol: in the ovary and adrenal glands for hormone production, in the spleen and bone marrow for blood cell membrane, and in the liver for bile production [29]. One has to assume that, in vivo, administration of bile acids could decrease the requirement of cholesterol, therefore the clearance of Ac-LDL by the liver endothelial cells. However, this limitation for targeting drugs to monocytes/macrophages, can be an advantage if the drug is released from endothelial cells to the liver, spleen and bone marrow cells that contain putative target macrophages for HIV. In addition, since it was recently shown that human liver endothelial cells are potential target cells for HIV and can play a role in the pathophysiology of AIDS [30], it can be proposed to use Ac-LDL for anti-HIV drug targeting to these cells. Concerning monocytes/macrophages, they are described as susceptible targets, persistent reservoirs for HIV and key immunoregulatory elements that control the extent of disease [31]. On the other hand, it has been described that efficient viral replication in monocytes or macrophages depends on both cell activation and differentiation [32],

known to increase the number of scavenger receptors for acetylated LDL at the cell surface [33,34]. Consequently, the use of the acetylated LDL receptor-mediated endocytosis for anti-HIV drug-targeting appears attractive. Finally, if the increase of acetylated LDL endocytosis in some CD14 monocytes reflects functional abnormalities of monocytes/macrophages in later stages of the disease, it is propitious to use acetylated LDL to carry drugs to these cells to restore or stimulate their functions.

In conclusion, this work leads to the consideration of LDL and acetylated LDL as potential candidates to carry anti-HIV drugs towards immune cells during AIDS.

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